Developmental Neurotoxicity of Chlorpyrifos Modeled *in Vitro*: Comparative Effects of Metabolites and Other Cholinesterase Inhibitors on DNA Synthesis in PC12 and C6 Cells

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The widely used organophosphate pesticide chlorpyrifos is a suspected neuroteratogen. In the current study, we compared the effects of chlorpyrifos and its major metabolites in two *in vitro* models, neuronotypic PC12 cells and gliotypic C6 cells. Chlorpyrifos inhibited DNA synthesis in both cell lines but had a greater effect on gliotypic cells. Chlorpyrifos oxon, the active metabolite that inhibits cholinesterase, also decreased DNA synthesis in PC12 and C6 cells with a preferential effect on the latter. Trichloropyridinol, the major catabolic product of chlorpyrifos, had a much smaller, but nevertheless statistically significant, effect that was equivalent in both cell lines. Diazinon, another organophosphate pesticide, also inhibited DNA synthesis with preference toward C6 cells, but was less effective than was chlorpyrifos. Physostigmine, a non-organophosphate cholinesterase inhibitor, was less effective than either chlorpyrifos or diazinon, but still caused significant inhibition of DNA synthesis in C6 cells. We also found that the addition of sera protected the cells from the adverse effects of chlorpyrifos and that the effect could be reproduced by addition of albumin. These results indicate that chlorpyrifos and other organophosphates such as diazinon have immediate, direct effects on neural cell replication, preferentially for gliotypic cells. In light of the protective effect of serum proteins, the fact that the fetus and newborn possess lower concentrations of these proteins suggests that greater neurotoxic effects may occur at blood levels of chlorpyrifos that are nontoxic to adults. *Key words*: C6 cells, chlorpyrifos, developmental neurotoxicity, diazinon, DNA synthesis, PC12 cells, physostigmine. *Environ Health Perspect* 109:909–913 (2001). [Online 20 August 2001]
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Despite recent restrictions on further production for home use, chlorpyrifos remains the most widely used organophosphate pesticide, and there is increasing concern over the potential consequences of fetal and childhood exposures (*1*). The acute toxicity of chlorpyrifos is mediated through inhibition of cholinesterase by the active metabolite chlorpyrifos oxon, but new evidence suggests that chlorpyrifos itself may influence brain cell replication and differentiation directly (*1–11*). Indeed, the greater toxicity of chlorpyrifos in juvenile animals cannot be explained solely by developmental differences in activities directed toward cholinesterase or neurotoxic esterase, nor do age-related increments in chlorpyrifos metabolism account for differential toxicity (*12*); immature animals actually recover more rapidly from cholinesterase inhibition, so measurements of cholinesterase activity other than in the immediate exposure period can give a misleading assessment of adverse effects (*7,13–15*).

Attempts have been made to model the mechanisms underlying the developmental neurotoxicity of chlorpyrifos *in vitro*, using either transformed neural cell lines (*1,6,11,16–18*) or cultures of immature brain tissue (*10,19,20*). Although transformed cells, such as the neuronotypic PC12 cell line, are typically less responsive to neurotoxins such as chlorpyrifos, they have proven especially useful in establishing cell replication as a major target because they maintain a fixed pattern of mitosis until differentiation is triggered by addition of trophic factors and deletion of serum (*1,6,8,17,18,21–23*). We recently found that replication of C6 cells, a gliotypic line, is also affected by chlorpyrifos (*18*); because glial development continues well into the postnatal period, this would in turn imply that the sensitive window for developmental neurotoxicity of chlorpyrifos could extend into childhood or even early adolescence.

The current work addresses several unanswered questions about the effects of chlorpyrifos on PC12 and C6 cells. First, to what extent does the antimitotic effect of chlorpyrifos extend to its major metabolites, chlorpyrifos oxon, the active inhibitor of cholinesterase, or trichloropyridinol, the catabolic product that is prominent in fetal brain after maternal chlorpyrifos exposure (*24*), which has been identified almost ubiquitously in urine samples of U.S. school children (*25,26*)? Second, are the effects of chlorpyrifos unique, or are they shared by other organophosphates such as diazinon, or by non-organophosphate cholinesterase inhibitors? Diazinon, like chlorpyrifos, has recently undergone major changes in its approved uses, with an initial phasing out of indoor use to be followed by reduced agricultural use (*25–27*). Third, to what extent might serum proteins protect the developing brain from the adverse effects of chlorpyrifos? It has recently been suggested that catabolic enzymes present in the serum could influence the neurotoxic effects of chlorpyrifos, at least *in vitro* (*11,18,28*), and other organophosphates show significant binding to plasma proteins (*29*). Fourth, to what extent might glia be affected preferentially to neurons? The standard growth conditions for these cell lines entail the addition of different sera and their associated proteins, so it is difficult to compare the actual vulnerability of gliotypic and neuronotypic cell lines (*1,18*). We have therefore compared the effects of chlorpyrifos as well as the other agents on gliotypic C6 and neuronotypic PC12 cells in experiments matching the incubation conditions so as to obviate any differential effects of serum proteins.

Materials and Methods

PC12 cells (American Type Culture Collection, CRL 1721; Duke University Comprehensive Cancer Center, Durham, NC) were seeded onto 60-mm poly-L-lysine-coated plates and grown for 48 hr at 37°C in 7.5% CO_2, using RPMI-1640 medium supplemented with 10% heat-inactivated horse serum, 5% inactivated fetal bovine serum, and 25 µg/mL penicillin–streptomycin (all from Gibco, Grand Island, NY). C6 cells (American Type Culture Collection, CCL 107) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 5% inactivated fetal bovine serum and 100 µg/mL penicillin–streptomycin. We conducted experiments toward the end of log-phase growth, when the cells had achieved about 70% confluence (*1,18*).

To initiate the measurement of DNA synthesis, we aspirated the medium and replaced it with medium containing 1 µCi/mL of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear Corp., Boston, MA), adding various concentrations of chlorpyrifos (Chem Service, West Chester, PA), chlorpyrifos oxon [U.S. Environmental Protection Agency (U.S.

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EPA), Research Triangle Park, NC], 3,5,6-trichloropyridinol (TCP; U.S. EPA), diazinon (Chem Service) or physostigmine (Sigma Chemical Co., St. Louis, MO). We dissolved the drugs in DMSO (Sigma) to produce a final DMSO concentration of 0.1% in the culture medium, and treated the control cultures with the vehicle; preliminary experiments verified the lack of effect of 0.1% DMSO. Depending on the specific experiment, the type of serum mixture and total serum concentration was changed at the same time as the radiolabel was added. These conditions, as well as the concentrations of test compounds, are described with each experiment and are commensurate with those used in earlier *in vitro* studies (*1,11,18,19,21–23,30–32*). One hour after adding [^{3}H]thymidine, we aspirated the medium and homogenized the cells in 3.5 mL of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid (TCA) and sedimented at 1,000 *g* for 15 min to precipitate macromolecules. The resultant pellet was washed once with TCA and then with 75% ethanol. The final pellet was then hydrolyzed in 1 M KOH overnight at 37°C and neutralized with 6 M HCl; the DNA was then precipitated with ice-cold 5% TCA and sedimented at 1,000 *g* for 15 min. The pellet from this final step was hydrolyzed in 5% TCA for 15 min at 90°C and resedimented, and an aliquot of the supernatant solution was counted for [^{3}H]thymidine incorporation. We assayed another aliquot for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of macromolecules by these techniques (*33*). We corrected incorporation values for the total amount of DNA to provide an index of DNA synthesis per cell.

Data analysis. Data are presented as means and standard errors, with differences among groups established by analysis of variance (ANOVA) followed by Fisher's protected least significant difference for individual comparisons. Significance was assumed at $p < 0.05$. To facilitate visual comparison across different cell types and incubation conditions, we present results as the percentage of the corresponding DMSO control group; however, statistical comparisons were based on the original data (log transformed whenever the variance was heterogeneous).

Results

In the first set of experiments, we exposed PC12 and C6 cells to chlorpyrifos or chlorpyrifos metabolites for 1 hr in the absence of serum, to obviate any potential protective effect of serum proteins (*11,18,28,29*), selecting a chlorpyrifos concentration (30 µM) previously found to cause robust but submaximal inhibition of DNA synthesis *in vitro* (*1,18*). Exposure of either cell type to chlorpyrifos elicited an immediate decrement in DNA synthesis, with a significantly greater effect on the gliotypic C6 cells than on neuronotypic PC12 cells (Figure 1). Equimolar concentrations of chlorpyrifos oxon also produced significant inhibition of DNA synthesis, again with C6 cells showing a greater effect than PC12 cells; however, chlorpyrifos oxon was also significantly less effective than was chlorpyrifos itself. At the same concentration, TCP produced less inhibition than chlorpyrifos or chlorpyrifos oxon but nevertheless still elicited a statistically significant decrement in DNA synthesis; in contrast to the effects of chlorpyrifos and chlorpyrifos oxon, the small effect seen for TCP did not display selectivity toward C6 cells. Given that the cells were exposed for only 1 hr, it is not surprising that there were no changes in total cell number, as evaluated by DNA content (data not shown); earlier work has demonstrated a lack of effect on cell viability at this chlorpyrifos concentration and duration of exposure (*1,18*).

We next compared the effects of chlorpyrifos to those of other cholinesterase inhibitors, again using equivalent concentrations (30 µM) of each compound (Figure 2). Both diazinon, an organophosphate, and physostigmine, a competitive cholinesterase inhibitor, caused significant inhibition of DNA synthesis in C6 cells, with the rank order chlorpyrifos > diazinon > physostigmine. For PC12 cells, diazinon caused a significant decrement, albeit smaller than the effect of chlorpyrifos, and physostigmine was ineffective.

Until this point, studies were conducted without the addition of sera to the incubation medium. Accordingly, we needed to determine if inclusion of serum combinations that are standard conditions for maintaining PC12 and C6 cell growth could influence the ability of chlorpyrifos to inhibit DNA synthesis (Figure 3). For these experiments, we reduced the chlorpyrifos concentration to 15 µM to allow for more sensitive detection of protective effects. At this lower chlorpyrifos concentration, we still observed robust inhibition of DNA synthesis in the absence of serum, and again the effect was greater in C6 cells. With addition of 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum to PC12 cells, the chlorpyrifos effect was substantially reduced but remained statistically detectable. In contrast, adding just the 5% heat-inactivated fetal bovine serum to C6 cells—the standard growth condition for this cell line (*18*)—failed to provide any protection whatsoever. It is interesting that the addition of serum alone had a differential effect on the two cell lines, with DNA synthesis increasing in C6 cells and decreasing in PC12 cells (data in legend to Figure 3). This may reflect differential effects of serum on cellular

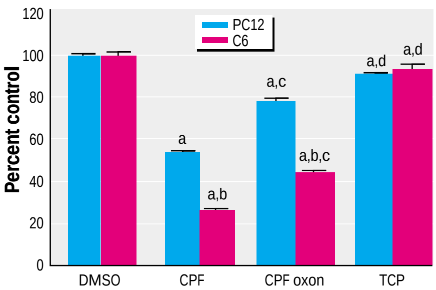

Figure 1. Effects of chlorpyrifos (CPF) and its metabolites (30 µM) on DNA synthesis in PC12 and C6 cells. Data represent means and standard errors obtained from 9–10 determinations for each treatment and cell type, presented as the percentage of corresponding control values: 17,400 ± 306 disintegrations per minute (dpm)/µg DNA for PC12 cells; 7,500 ± 80 for C6 cells. DNA content was 42 ± 4 µg/dish for PC12 cells and 29 ± 1 for C6 cells, and was unaffected by the treatments (data not shown). ANOVA: treatment, $p < 0.0001$; treatment × cell type, $p < 0.0001$. Treatment with chlorpyrifos, chlorpyrifos oxon, or TCP elicited significant reductions in DNA synthesis in both cell types (a). For chlorpyrifos and chlorpyrifos oxon, the effects were significantly greater in C6 cells than in PC12 cells (b). The effect of chlorpyrifos oxon was significantly smaller than that of chlorpyrifos in either cell type (c), and the effect of TCP was significantly smaller than either of the other two treatments (d).

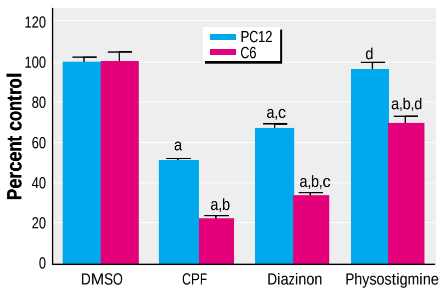

Figure 2. Effects of cholinesterase inhibitors (30 µM) on DNA synthesis in PC12 and C6 cells. Data represent means and standard errors obtained from 10 determinations for each treatment and cell type, presented as the percentage of corresponding control values: 17,000 ± 600 dpm/µg DNA for PC12 cells; 6,020 ± 240 for C6 cells. DNA content was 31 ± 2 µg/dish for PC12 cells and 28 ± 1 for C6 cells, and was unaffected by the treatments (data not shown). ANOVA: treatment, $p < 0.0001$; treatment × cell type, $p < 0.0001$. Treatment with chlorpyrifos (CPF) or diazinon elicited significant reductions in DNA synthesis (a) in both cell types, whereas physostigmine affected only C6 cells. For all three treatments, the effects were significantly greater in C6 cells than in PC12 cells (b). The effect of diazinon was significantly smaller than that of chlorpyrifos in either cell type (c), and the effect of physostigmine was significantly smaller than either of the other two treatments (d).

metabolism, because changes in serum conditions can also elicit cell differentiation and/or apoptosis in these cell lines (*11,34–36*).

It was not clear from these results alone whether the lack of protective effect of serum on C6 cells represented a different underlying mechanism for chlorpyrifos action compared to PC12 cells, whether the key ingredient was the horse serum (which was included for normal growth of PC12 cells but not C6 cells), or whether simply the higher total serum protein concentration from the combined sera represented the critical factor. Previous work had suggested that, despite heat inactivation, horse serum retains catabolic capabilities toward chlorpyrifos oxon, and thus potentially toward chlorpyrifos (*11*). Accordingly, we performed further studies with C6 cells, comparing different serum mixtures (Figure 4). As before, 5% fetal bovine serum had no significant protective effect. However, addition of the PC12 serum mixture to C6 cells (10% horse serum + 5% fetal bovine serum) completely protected the C6 cells from the effects of chlorpyrifos. Surprisingly, a similar protective effect could be obtained simply by adding albumin in the same total protein concentration as the serum mixture.

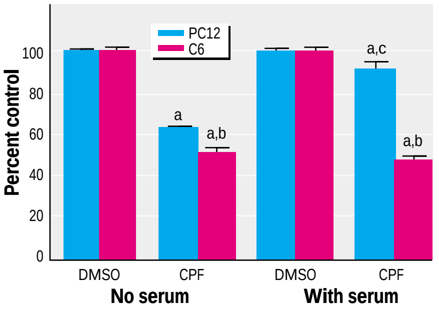

Figure 3. Effects of standard serum conditions on the ability of chlorpyrifos (CPF; 15 µM) to inhibit DNA synthesis in PC12 and C6 cells. PC12 cells were grown in the presence of 10% horse serum + 5% fetal bovine serum; C6 cells were grown with 5% fetal bovine serum. Serum was then deleted (no serum) or continued (with serum) during the chlorpyrifos exposure period. Data represent means and SEs obtained from 9–11 determinations for each treatment and cell type, presented as the percentage of corresponding control values: 13,600 ± 120 dpm/µg DNA for PC12 cells without serum, 10,700 ± 150 with serum ($p < 0.0001$); 6,600 ± 100 for C6 cells without serum, 8,200 ± 300 with serum ($p < 0.0002$). DNA content was 27 ± 1 µg/dish for PC12 cells and 33 ± 1 for C6 cells, and was unaffected by the addition of serum or by the chlorpyrifos treatment (data not shown). ANOVA: treatment, $p < 0.0001$; treatment × cell type, $p < 0.0001$; treatment × serum, $p < 0.0001$; treatment × cell type × serum, $p < 0.0001$. Treatment with chlorpyrifos elicited significant reductions in DNA synthesis (a) in both cell types in the absence of serum; the effect was greater on C6 than on PC12 cells (b). The addition of serum prevented most of the effect of chlorpyrifos on PC12 cells (c) but did not protect C6 cells.

Discussion

The current results are consistent with the concept that chlorpyrifos exerts antimitotic actions on developing neural cells independently of cholinesterase inhibition (*5,9,11*). First, we found that chlorpyrifos was more effective than chlorpyrifos oxon, despite the fact that the latter is a far more potent cholinesterase inhibitor. Second, physostigmine, a non-organophosphate cholinesterase inhibitor, was totally ineffective in inhibiting DNA synthesis in PC12 cells and was less effective than chlorpyrifos in C6 cells. These results also agree with a recent study in which chlorpyrifos, but not chlorpyrifos oxon or physostigmine, disrupted maturation of sea urchin embryos during the specific period in which development is regulated by neurotrophic factors (*37*). Although our studies do not address the specific molecular or cellular mechanism by which chlorpyrifos disrupts DNA synthesis, the rapidity of the effect, with onset within as little as 1 hr, is consistent with postulated actions at the level of transcriptional events mediating cell division and differentiation (*6,8,18*).

We found that gliotypic C6 cells are much more sensitive than neuronotypic PC12 cells. Although we had previously postulated preferential targeting of glia (*18*), there were potential confounds in our comparing cultures with different combinations of sera in the medium, and the current results indicate that the greater sensitivity of

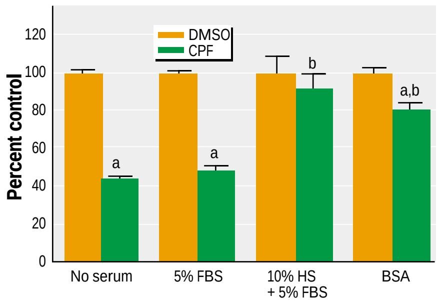

Figure 4. Effects of different serum mixtures on the ability of chlorpyrifos (15 µM) to inhibit DNA synthesis in C6 cells. C6 cells were grown with 5% fetal bovine serum (FBS) and then serum was either deleted or continued (no serum) during the chlorpyrifos exposure period, or a different serum mixture used for PC12 cells was added [10% horse serum (HS) + 5% FBS], or bovine serum albumin (BSA) was added to achieve the same protein concentration as in the PC12 serum mixture. Data represent means and standard errors obtained from 5–20 determinations for each combination of treatment and serum condition, presented as the percentage of corresponding control values (see legend to Figure 3). ANOVA: treatment, $p < 0.0001$; treatment × serum, $p < 0.0001$. Treatment with chlorpyrifos elicited significant reductions in DNA synthesis (a) in the presence or absence of 5% FBS. Addition of 10% HS + 5% FBS protected the cells from chlorpyrifos (b) and a similar protection was obtained with BSA.

C6 cells is present even in the absence of serum proteins. Adverse effects of chlorpyrifos on glial cell replication are of critical importance in defining the sensitive period for effects on central nervous system development. Glia provide nutritional, structural, and homeostatic support that are essential to architectural modeling of the brain (*38–42*), and because glial development continues well into the postnatal period, glial targeting implies a prolonged vulnerability, extending into childhood. In keeping with this interpretation, chlorpyrifos administration *in vivo* inhibits DNA synthesis and causes loss of brain cells during gliogenesis (*2,4,43*), with maximal effects on neural function appearing during peaks of glial development (*3,5,10,43,44*). In aggregating brain-cell cultures, chlorpyrifos affects glial markers, again unrelated to cholinesterase inhibition (*10*). The present results thus confirm conclusively that chlorpyrifos, rather than its active metabolite, chlorpyrifos oxon, is the primary agent in these effects.

Our findings also extend the results to another organophosphate, diazinon, suggesting that chlorpyrifos is not unique in its adverse effects on neural cell replication. Like chlorpyrifos, diazinon showed immediate onset of effects and preferential actions on gliotypic cells. Similarly, diazinon has been shown to disrupt neurodevelopment in aquatic species (*45,46*) and elicits dysmorphogenesis in sea urchins during the phase in which neurotrophic factors control development (*47*). The effects, however, are not shared by non-organophosphate pesticides: We previously found dieldrin to be ineffective in the sea urchin model (*37*), and physostigmine, a carbamate, was much less effective, as found here. The smaller effect of physostigmine is nevertheless of some additional interest, especially because inhibition was seen only in C6 cells and not in the neuronotypic cells. Given the greater effect of chlorpyrifos compared to chlorpyrifos oxon, it is highly unlikely that inhibition of cholinesterase per se is responsible for the effect of physostigmine, and it certainly could not explain differential sensitivity of the two cell lines. Cholinesterase plays a nonenzymatic role in the proliferation of glial cells (*48,49*), and it is possible that physostigmine affects structural aspects of the cholinesterase molecule in a manner different from that of chlorpyrifos or chlorpyrifos oxon; further study will be needed to characterize any such effect. Physostigmine also can interact directly with nicotinic cholinergic receptors (*50*), but one would then expect PC12 cells, which are enriched in these receptors, to be targeted far more than C6 cells, which are not, rather than the converse, as found here.

A major limitation of the current study is the use of transformed cell lines, which provide the advantage of a uniform cell population undergoing synchronous replication, but which are less responsive to neurotoxins such as chlorpyrifos (*1,6,8,11,17,18,21,22*). Accordingly, whereas potencies of different chemicals can be compared in these systems, it is difficult to relate the absolute levels required for these actions to safety thresholds *in vivo*. Given the requirement that serum proteins be excluded from the medium for comparisons of PC12 and C6 cells (discussed below), the determinations here also are limited to short-term studies, because the prolonged absence of sera terminates cell division, initiates differentiation into several phenotypes, and eventually can elicit apoptosis (*11,34–36*). When studies are conducted over a much longer time frame, considerably lower concentrations of chlorpyrifos evoke inhibition of DNA synthesis and other neurotoxic events culminating in cell damage and loss (*1,11,18*).

Despite these constraints, *in vitro* systems allow for the dissection of specific cellular mechanisms underlying developmental neurotoxicity, in this case the direct inhibition of DNA synthesis as a separable event from cholinesterase inhibition. Furthermore, these results can be combined with *in vivo* data to give insight into unexpected consequences. Thus, combined with pharmacokinetic information (*51*), our finding of a slight inhibitory activity of TCP on DNA synthesis assumes greater importance. TCP accumulates in high concentrations in fetal brain after maternal chlorpyrifos administration (*51*) and is also the major chlorpyrifos residue in children (*25,26*); the smaller *in vitro* effect of TCP than chlorpyrifos found here may thus represent a much more important effect *in vivo*. On the other hand, the fact that chlorpyrifos oxon was more potent than TCP and less potent than chlorpyrifos probably indicates that the oxon does not contribute significantly to mitotic inhibition and loss of cells *in vivo*, in light of the much lower concentrations and short biologic half-life of this active metabolite (*51*). Unlike chlorpyrifos, TCP did not show a preferential effect toward gliotypic cells, implying that this supposedly inactive metabolite (*52*) may in fact have a more ubiquitous effect on neural cell development, targeting neurons and glia equally. Indeed, TCP, like chlorpyrifos, has been shown to inhibit neurite outgrowth in neuronotypic PC12 cells (*11*). Accordingly, future studies should examine the potential developmental neurotoxicity of TCP.

Finally, our results address the important issue of differential neurotoxicity of chlorpyrifos in the developing brain compared to the mature brain. Previous work with PC12 cells suggested that horse serum, although heat-inactivated, nevertheless might maintain sufficient A esterase and carboxylesterase activity to hydrolyze chlorpyrifos oxon and thus provide protection from its adverse effects (*11*). Although the ability of these enzymes to hydrolyze chlorpyrifos is poorly characterized, we were indeed able to show that addition of inactivated horse serum to the medium protected PC12 cells from the inhibition of DNA synthesis evoked by chlorpyrifos. Furthermore, although horse serum is not ordinarily required to maintain growth of C6 cells, the combination of 10% horse serum and 5% fetal bovine serum was able to protect these cells from chlorpyrifos as well; the lower concentrations of fetal bovine serum alone, typically used with C6 cells, did not protect the cells from chlorpyrifos. Surprisingly, though, we found equivalent protection when we added albumin to achieve the same total protein concentration as provided by the sera. This implies that the protective effect is not an artifact of residual enzymatic activity after heat inactivation but most likely represents binding of chlorpyrifos to serum protein. The fact that serum proteins can bind chlorpyrifos and thus protect developing neural cells may be of key importance for fetal and neonatal neurotoxicity, because their plasma protein binding is deficient relative to the adult (*53*). Thus, at the same plasma concentration of chlorpyrifos, a greater proportion of the compound will be biologically active in the immature organism. Accordingly, the development of binding proteins may prove to be as important for organophosphate-induced developmental neurotoxicity as serum esterase activity (*54–57*).

In conclusion, chlorpyrifos and other organophosphates such as diazinon have immediate, direct inhibitory actions on DNA synthesis and hence on neural cell replication, with preferential targeting of gliotypic cells. The active metabolite, chlorpyrifos oxon, is less effective than chlorpyrifos, indicating that the antimitotic effects are separable from inhibition of cholinesterase. However, additional effects may be contributed by the supposedly inactive major metabolite, TCP. In light of the protective effect of serum-binding proteins, the fact that the fetus and newborn possess lower concentrations of these proteins suggests that greater neurotoxic effects can occur at the same blood levels of chlorpyrifos that are nontoxic to adults. *In vitro* cultures of neural cells can thus permit the determination of the mechanisms underlying developmental neurotoxicity of pesticides, as well as providing a rapid screening procedure.

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